Journal of Microbiology Research 2012, 2(2): 41-46
DOI: 10.5923/j.microbiology.20120202.07

Diversity and Antibacterial Screening of Actinomycetes from Javadi Hill Forest Soil, Tamilnadu, India

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Abstract Actinomycetes from diverse environments are known to produce novel antibacterial and antifungal substances. Herein we report isolation of antibiotic producing actinomycetes from forest soil of ancient human inhabited Javadi hill, Tamilnadu, India, an environment that is under explored. Thirty six isolates were obtained from five soil samples using nalidixic acid and nystatin supplemented starch casein agar and actinomycete isolation agar medium. These colonies NLO were characterized based on their mycelium structure, colour and arrangement of spores on the mycelium. Further they were evaluated for their antimicrobial activity against a range of pathogenic resistant bacteria including *Escherichia coli* (MTCC 739), *Bacillus cereus* (MTCC 1272), *Staphylococcus aureus* (MTCC 1144), *Pseudomonas aeruginosa* (MTCC 1688), *Proteus mirabilis* (MTCC 1425) and *Klebsiella pneumonia* (MTCC 109) adopting agar plug method and confirmed by cross streak method.

Keywords Actinomycetes, antibacterial activity, antagonistic activity, pathogens

1. Introduction

Microorganisms are virtually an unlimited source of novel substances with many therapeutic applications and consequently their secondary metabolite screening for pharmaceutically significant novel antibiotic and non - antibiotic compounds and drug lead molecules has assumed greater attention in recent times. Many soil-inhabiting bacteria are known to produce secondary metabolites that can suppress microorganisms competing for the same resources[1].

Actinomycetes are the most commonly distributed microbes in nature which largely inhabits the soil environment[2]. They form the dominant and significant group among the soil microbial community and comprise about 50% of the uncultivable soil microbes. They play a major role in the recycling of organic matter, production of novel pharmaceuticals, nutritional materials, cosmetics, enzymes, antitumor agents, enzyme inhibitors, immune modulators, and vitamins. Numerous naturally occurring antibiotics have been discovered from actinomycetes ever since the discovery of Selman Waksman's streptomycin from this group and several studies signify their noteworthy antibiotic production[3]. Further, about two thirds of known significant naturally occurring antibiotics are actinomycete derived one and are the prominent candidates receiving number of product and processes related patents.

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Published online at http://journal.sapub.org/microbiology

Though ecological studies on soil actinomycetes from various habitats including grasslands, beach sands, underground caves, rice-paddies, orchards and sub-glacial ice of Antarctica were reported, only few reports are available on forest soil actinomycete communities[4]. These ubiquitous organisms are deemed to have a preference over the soil constituents such as humus, litter, dung and even rock surfaces. In fact actinomycetes are the dominant microflora showing viable counts reaching 10^6 per gram of dry weight soil in relatively dry, humic, and calcareous soils,[5].

The Eastern Ghats is a discontinuous range of mountains along the east coast of India, located between 10º 05' and 22º 30' N latitude and 76º 23' and 86 º 50' E longitudes in north east to southwest strike, which is broken and comprises many viz. Shevaroys, Kalrayan, Chitteri, Kollimalai, Pachchimalai hills of Tamilnadu. Very little information is available on the actinomycetes population of these hills including those from Kollimalai[6] and Pachamalai[7].

Wealth of information/ knowledge is available on the early inhabitants of Javadi hills, a place inhabited by human being even during Stone Age. Claims for supporting this are available in the form of 4000 years old Stone Age tribal caves. There are traces of evidence depicting the presence of Chitra Kullers before the invasion of present day outsiders. The rock houses still exist at Chepli above Pattaraikadu giving an affirmation that they might be kullers or the early tribes who lived as hunters. The glory of Javadi hills was prized even in the Patthu pattu, one of the earliest classical language Tamil literatures.

The Javadi Hills, the largest of Eastern Ghats forest consists of dray mixed deciduous to thorny shrubs with occa-

sional patches of dray ever green growth. The maximum temperature go up to 44.4 ^{0} C during the summer month May and minimum temperature falls to 11.7^0 C during the winter month January. The average rain fall is about 886mm. Amirthi forest is situated under the Javvadu/Javadi Hills of Tellai across Amirthi River contains a wide variety of flora and fauna. The soil is highly rich in organic matter and suitable for the growth of microorganisms[8].

Although there are a number of reports on distribution and traditional uses of medicinal plants from these early human inhabitated areas are readily available, data on their microbial resources are scarce, imprecise, and not well documented. Due to uniqueness, large geographic variation, different soil types and their contents of this forest, it is quite likely that there is vast distribution of antibiotic producing actinomycetes in this environment. The present study was carried out to screen the most assured antimicrobial compounds producing actinomycetes from these unexplored soils for possible harnessing of potential antibiotics from the actinomycetes towards combating highly resistant pathogenic bacteria.

2. Materials and Methods

2.1. Sampling Area and Sample Collection

The Javadi Hills, the largest of Eastern Ghats are located in Vellore district (Tamilnadu, India) at an altitude of 300–1000m above sea level (longitude 78° 40' E; latitude 12° 40' N), in North to South direction, covering a distance of 80 km in width and 32 km in length. Its average height is about 214 m, bisected into eastern and western sections by the Cheyyar and Agaram rivers. The centre of the Javadi hills consists of extensive undulating plateau with large valleys ranging from 500–800 metres. Soil samples were collected from this hill at 10-15 cm depth of the soil[9], air-dried for 1 week[10], crushed, and sieved. The sieved soils were then used for isolation of actinomycete.

2.2. Isolation of Actinomycetes Isolates

The actinomycetes from crushed and sieved soil samples were isolated as described previously[11] employing serial dilution and spread plate technique. One gram of soil sample was transferred into 9 ml of sterile double distilled water (10^1) and serially diluted up to 10^{-5} dilutions using each 9 ml of sterile double distilled water blanks. Hundred microliter of diluted soil sample from 10^{-3}, 10^{-4} and 10^{-5} dilutions was spreaded on starch casein agar[12] plates supplemented with nalidixic acid (20µg/ml) and nystatin (100µg/ml) as well as actinomycete isolation agar (M490, HiMedia, Mumbai, India). Plating was done in triplicate and all the plates were incubated at 28ºC for one week. The individual colonies were selected based on morphology and purified by inoculation onto ISP-2 (International *Streptomyces* Project) agar plates which were incubated for 7–14 days at 28ºC. Morphologically distinct colonies were selected for further studies.

2.3. Microorganisms Used

The bacterial strains of *Escherichia coli* (MTCC 739), *Bacillus cereus* (MTCC 1272), *Staphylococcus aureus* (MTCC 1144), *Pseudomonas aeruginosa* (MTCC 1688), *Proteus mirabilis* (MTCC 1425) *Klebsiella pneumonia* (MTCC 109) as well as *Candida albicans* obtained from microbial type culture collection (MTTC) Chandigarh, India were used in this study.

2.4. Determination of Bacterial Antagonistic Activity

Antagonistic activities of the selected isolates were tested by adopting agar plug method[13]. The isolates were inoculated onto ISP2 agar plates and were allowed to grow restricted towards one end of the plate for 10 days. Later 5 mm diameter core agar plug were removed from the grown cultures and the surface growth on agar was removed with sterile knife to allow remaining of only the diffused microbial metabolites in the agar plugs. The agar plugs were placed onto the nutrient agar plate which was previously swabbed with the test pathogens. All the plates were then incubated at 37º C for 24 h. After incubation, antimicrobial activity indicated by the formation of an inhibition zone surrounding the agar plug was measured. The absence of an inhibition zone around the plugs indicated a negative result. The observed growth inhibitory activities of the isolates were further confirmed by cross streak test. The isolates showing significant inhibitory activities were inoculated on modified nutrient glucose agar (MNGA) plates by single streak in the center and incubated at 28^{o}C for four days. The previously used test pathogenic organisms were inoculated perpendicular to the actinomycetes growth and incubated at 37^{o}C for 24 h. The antagonistic activities of the isolate were confirmed by their growth inhibition. Isolates showing significant inhibitory activity against at least two pathogenic test organisms were further selected for secondary screening.

2.5. Determination of Bioactive Compound production and Their Inhibitory Activity

The bioactive substance producing ability of the isolates that showed antagonistic activity was determined by a method described by Mohanraj et al.[14]. Briefly, the selected bacterial antagonistic isolates were grown in submerged fermentation condition by adopting shake flask method. Initially 10% of culture inoculum was transferred into each 100 ml of ISP2 broth and incubated in a rotary shaker with 95 rpm at 28^{0}C for 7 days. The cell free supernatant was obtained and 100 µl was poured into 5mm well made in nutrient agar (NA) plates. The NA plates were previously inoculated with test organism cultures as described. After incubation the diameter of the zone of inhibition was measured in millimeter and recorded. The isolates were designated as BKM (Bheemakulam), KUR (Kavalur), PMR (Palamarathur), VPR (Veerappanur) as well as JMR (Jamunamathur) according to the place of collection and

preserved in 15% glycerol (v/v) slants at -20^{o}C.

3. Results and Discussion

Antibiotics are an indispensable part of modern medicine. The emergence of antibiotic-resistance among pathogenic bacteria is apparently certain, and results, within a few decades, in decreased efficacy and withdrawal of the antibiotic from general practice. Hence for the continuance of modern medicine in its present form, novel families of antibiotics must be available in the market at regular intervals[15]. Many important bioactive compounds of high commercial value were obtained from actinomycetes and the screening is being continued for deriving new bioactive compounds especially novel antibiotics active against resistant organisms. This endeavour was undertaken with an objective of identifying culturable new actinomycetes having novel anti-bacterial activity against the resistant pathogenic bacteria from the virgin soils of Javadi hills.

Table 1. Morphology and colour of aerial mycelium of Actinomycetes isolates

S.No	Isolates Name	Colour of colony	Colony texture	Morphology of hyphae
1	BKM -1	Yellow	Fine substrate mycelium	long fragmented
2	BKM -2	Grey	Powdery colony	Extended spiral
3	BKM -3	Brown	Waxy colony	long fragmented
4	BKM -4	Grey-white	Powdery colony	Extended spiral
5	BKM -5	Grey	Powdery colony	Straight Un fragmented
6	BKM -6	Grey	Powdery colony	Extended spiral
7	BKM -7	Grey-white	Powdery colony	Extended spiral
8	KUR-8	Yellow	Fine substrate mycelium	long fragmented
9	KUR -9	Grey	Powdery colony	Straight -Un fragmented
10	KUR -10	Reddish brown	Fine substrate mycelium	Straight -Un fragmented
11	KUR -11	Grey	Powdery colony	Extended spiral
12	KUR -12	Yellow brown	Waxy colony	Straight looped end
13	PMR-13	Reddish brown	Fine substrate mycelium	Straight -Un fragmented
14	PMR -14	Orange	Fine substrate mycelium	long fragmented
15	PMR -15	Red	Fine substrate mycelium	Extended spiral
16	PMR -16	Grey	Powdery colony	Extended spiral
17	PMR -17	Yellow	Waxy colony	long fragmented
18	PMR -18	Grey-white	Powdery colony	Extended spiral
19	PMR -19	Grey	Powdery colony	Extended spiral
20	PMR -20	Grey	Powdery colony	Extended spiral
21	PMR -21	Reddish brown	Fine substrate mycelium	Straight -Un fragmented
22	PMR -22	Grey yellow	Powdery colony	Straight looped end
23	VPR-23	Grey	Powdery colony	Straight -Un fragmented
24	VPR -24	Yellow	Fine substrate mycelium	long fragmented
25	VPR -25	Yellow	Fine substrate mycelium	long fragmented
26	VPR -26	Reddish brown	Fine substrate mycelium	Straight -Un fragmented
27	VPR -27	Orange	Fine substrate mycelium	long fragmented
28	VPR -28	Grey violet	Powdery colony	Extended spiral
29	VPR -29	Grey	Powdery colony	Extended spiral
30	VPR -30	Red	Fine substrate mycelium	Extended spiral
31	JMR-31	Yellow	Fine substrate mycelium	long fragmented
32	JMR -32	Reddish brown	Fine substrate mycelium	Straight -Un fragmented
33	JMR -33	Grey pink	Powdery colony	Straight -Un fragmented
34	JMR 34	Grey-white	Powdery colony	Extended spiral
35	JMR 35	Brown	Fine substrate mycelium	long fragmented
36	JMR 36	Grey	Powdery colony	Extended spiral

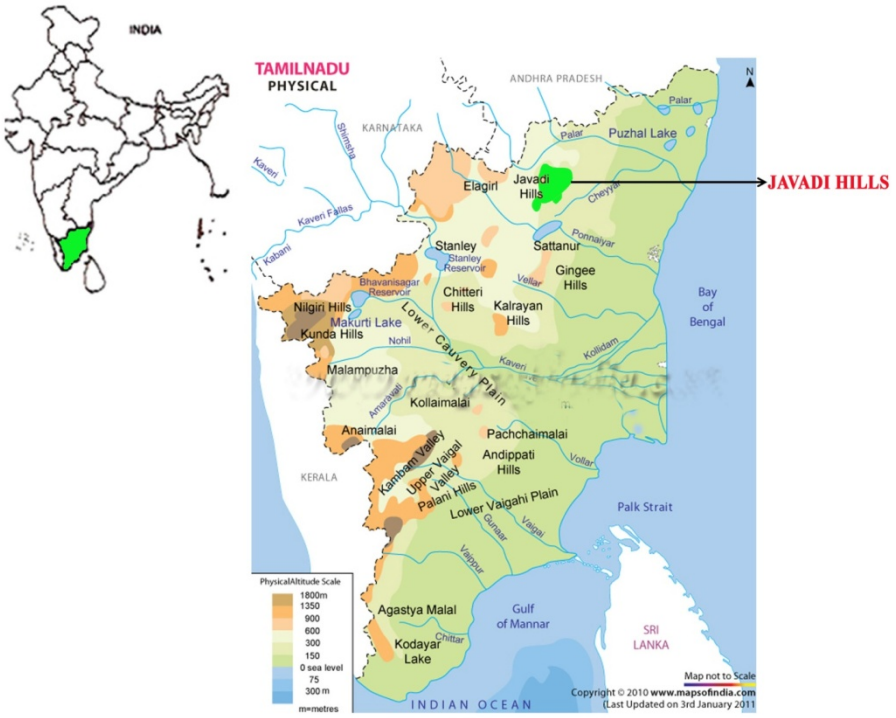

Figure 1. Schematic map showing the location of Javadi hills, Tamilnadu, India(Courtesy:www.mapsof India)

Table 2. Screening for antagonistic activity

S.No.	Isolates	EC	BS	SA	PA	PM	KP	CA
1	BKM-1	22	0	0	11	0	18	13
2	BKM-2	0	13	15	0	0	0	0
3	BKM-3	17	0	13	12	10	11	0
4	BKM-4	24	16	18	20	14	25	0
5	BKM-5	0	15	15	0	0	0	12
6	BKM-6	0	14	11	0	0	0	11
7	BKM-7	15	0	0	14	16	17	0
8	KUR-8	18	0	0	13	0	22	12
9	KUR-9	0	16	18	0	0	0	0
10	KUR-10	8	11	9	11	12	10	0
11	KUR-11	0	15	13	0	0	0	15
12	KUR-12	8	0	16	14	12	23	21
13	PMR-13	0	0	0	13	0	9	0
14	PMR-14	0	0	0	0	0	0	0
15	PMR-15	0	0	0	0	0	0	0
16	PMR-16	0	15	14	0	0	0	12
17	PMR-17	21	0	0	12	8	7	11
18	PMR-18	19	14	16	20	12	16	0
19	PMR-19	0	12	15	0	0	0	0
20	PMR-20	0	0	0	0	0	0	12
21	PMR-21	8	11	11	12	9	10	8
22	PMR-22	0	0	0	12	15	23	11
23	VPR-23	9	0	0	12	16	14	13
24	VPR-24	14	0	0	13	0	0	11
25	VPR-25	19	0	0	0	0	0	9
26	VPR-26	11	0	0	10	0	13	0
27	VPR-27	12	0	0	0	21	14	14
28	VPR-28	0	0	0	0	0	0	12
29	VPR-29	9	15	16	0	0	11	0
30	VPR-30	0	0	0	0	0	0	10
31	JMR-31	14	0	0	13	8	7	14
32	JMR-32	0	0	0	18	9	10	0
33	JMR-33	12	13	9	0	0	8	0
34	JMR-34	22	18	19	21	20	11	9
35	JMR-35	15	0	10	16	9	14	0
36	JMR-36	9	0	0	12	16	14	13

Note: EC-Escherichia coli, BS-Bacillus subtilis, SA-Staphylococcus aureus, PA-Pseudomonas aeruginosa, PM-Proteus mirabilis, KP-Klebsiella pneumonia, CA-Candida albicans

The forest soil samples collected from five different locations of Javadi hills upon inoculation on nutrient media yielded plenty of actinomycetes having different cultural and morphological characters. From among them thirty six actinomycetes strains were selected based on the colony morphology on starch casein agar and actinomycetes isolation agar medium. All of these collected strains were suspected to be actinomycetes since SCA medium supplemented with nalidixic acid (20 mg/ml) and nystatin (100 mg/ml) is a selective medium which inhibit the growth of other bacteria and fungi. Selective medium with nalidixic acid is found to be efficient for soil actinomycetes isolation and it is recommended for screening organisms producing new antibiotics[16]. Further this medium is very specific for the isolation of actinomycetes, as only organism (mostly actinomycetes) capable of degrading the polymers in the medium will be able to grow.

Five to ten colonies from each plate were selected based on colony appearance. Colonies having characteristic features such as powdery appearance with convex, concave or flat surface and color ranging from yellow, grey, brown, white, red and pinkish were selected. Colonies observed on 1st and 2nd day were eliminated because actinomycetes are considered as slow grower[17]. Furthermore, bacterial configuration same as actinomycetes were accepted from Gram staining. All the thirty six selected isolates were examined microscopically for their morphological characteristics and for their actinomycetes cultural characteristics (Table.1).

The bacterial antagonistic activities of all selected isolates determined against pathogenic bacteria by agar plug method and confirmation by cross streak method revealed the significant antibacterial and antifungal activities of most of the isolates (Table2). All the thirty six actinomycetes were screened for their antibacterial activity against five species of bacteria and one species of fungal pathogens. Antagonistic activities of actinobacterial isolates were tested by adopting agar plug method. Isolate BKM-4 showed antibacterial activities against four species of bacterial pathogens except *Candida albicans*. But PMR-18 strain showed antibacterial activities against both bacterial and fungal pathogens. Moreover six isolates BKM-1, BKM-4, BKM-7, KUR-8, KUR-12, PMR-17, JMR-34 and PMR-18 were found to have high activity against *Escherichia coli, Pseudomonas aeruginosa,* and *Klebsiella pneumoniae* tested for sensitivity. Ten different types of actinomycetes were found to have high antagonistic activity. Especially BKM-4 soil produces an intense antagonism. Among the screening methods used, agar plug, crowded plate, agar overlay, and cross streak used for the antagonistic activity detection, the agar plug method allowed utilization of very small amount of medium for both cultivation as well as bioactive compounds production besides antimicrobial activity detection of more number of actinomycetes against wide range of pathogens with less cost[14].

4. Conclusions

Considering the outcome of the present investigation, it was concluded that Javadhu hills is a rich source for deriving economically important Actinomyces. The antibiogram results indicated that the hill soils are source for hyperactive actinomycetes antagonistic against the pathogenic bacteria and fungi. The widest activity spectrum and the largest inhibition zones were shown by strains BKM-4, JMR-34 and PMR-18 while the first exhibited the best performance. Thus there is definite scope for bioprospecting of antagonistic actinomycetes from Javadi hill forest soil ecosystem once appropriate further studies are undertaken.

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